ORGANIC LETTERS

2002
Vol. 4, No. 22
3863−3866

Rational Design of Vitamin D_3 Analogues Which Selectively Restore Activity to a Vitamin D Receptor Mutant Associated with Rickets

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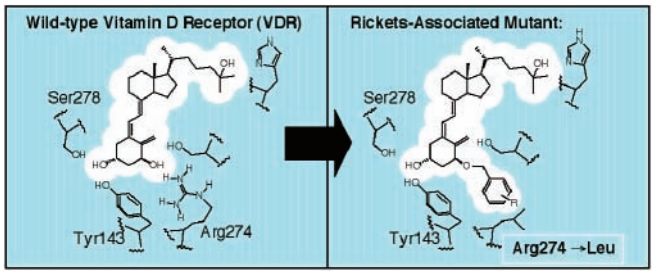
Received August 7, 2002

ABSTRACT



Vitamin D_3-resistant rickets (VDRR) is associated with mutations to the Vitamin D receptor (VDR) which effect ligand-dependent transactivation. Some VDRR associated mutants directly disrupt ligand binding. Using the reported VDR-1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) cocrystal structure, three $1,25(OH)_2D_3$ analogues were designed to uniquely complement the rickets associated mutant VDR(Arg274→Leu). The three analogues were 17 to 286 times more potent than $1,25(OH)_2D_3$ with the mutant in cell-based assays and did not substantially activate cellular calcium influx.

A few recent examples have shown that small molecules (MW < 600) can restore activity to mutationally impaired proteins that are associated with genetic disease. For example, compounds have been discovered which can help stabilize mutant forms of p53 associated with cancer,[1] or recover activity to mutated forms of nuclear hormone receptors associated with refractory forms of prostate cancer,[2] resistance to thyroid hormone (RTH),[3] and type II rickets.[4] These examples illustrate that small molecules may be used to restore activity to at least a subset of genetic mutations and suggest a potentially new pharmacological approach to the treatment of genetic disease. Thus far, mutant-complementing molecules have almost exclusively been discovered by screening existing compounds and compound libraries. In this study, we evaluate the ability of structure-based design to custom design hormone analogues for a vitamin D receptor (VDR) mutation associated with vitamin D resistant rickets (VDRR).

The nuclear and steroid hormone receptors (NHR's) comprise a superfamily of ligand-dependent transcriptional regulators that control the expression of specific eukaryotic genes involved in development and homeostasis.[5,6] The NHRs bind to specific DNA sequences (response elements)

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10.1021/ol0266931 CCC: $22.00
Published on Web 09/28/2002

that are part of the promoters of hormone responsive genes and activate gene expression in a ligand-dependent manner.

A diverse class of human genetic diseases is associated with mutations to NHRs, including androgen insensitivity syndrome (androgen receptor), resistance to thyroid hormone (RTH, thyroid hormone receptor), diabetes mellitus (peroxisome proliferator activated receptor), rickets (VDR), Cushings disease (glucocorticoid receptor), and certain forms of prostate and breast cancer (androgen and estrogen receptors).[7,8] Many of these disease-associated mutations involve amino acids that lie in and around the hormone binding pocket and directly affect hormone binding and hormone-dependent transactivation function.

Recent work from our lab has demonstrated that rational molecular design can be used to generate a potent and selective thyroid hormone analogue, which selectively activates an RTH-associated mutant of TRβ over its α subtype (TRα), which is associated with cardiac function.[9] Efforts to complement other RTH-associated mutations of TR mutations are currently ongoing. In this study we explore the generality of our structure-based design approach to complement mutations in a related NHR family member.

In response to 1,25-dihydroxy vitamin D_3 (1,25$(OH)_2D_3$), the nuclear vitamin D receptor (VDR) regulates genes associated with phosphate and calcium homeostasis and bone maintenance.[10,11] Several studies have also demonstrated the presence of a second membrane−associated 1,25$(OH)_2D_3$ receptor, which is responsible for rapid hormone-dependent responses including stimulating cellular calcium influx and activation of PKC.[12]

Human Vitamin D-Resistant Rickets (VDRR) is associated with mutations to the nuclear VDR, which result in high serum 1,25$(OH)_2D_3$ levels and severe bone underdevelopment and alopecia. Presently, over 20 VDR mutations associated with VDRR have been reported.[13] Two of these mutations, Arg274→Leu and His305→Gln, involve residues that directly contact 1,25$(OH)_2D_3$ in the normal ligand−receptor complex. The Arg274→Leu mutation results in a net loss of a hydrogen bond to the 1α-hydroxyl of 1,25-$(OH)_2D_3$ and a greater than 1000-fold decrease in hormone-responsive transactivation compared to "wild-type" receptor, VDR(wt) (EC_{50}VDR(wt) = 2nM, EC_{50}VDR(R274L) = >2000 nM). Patients with weakly refractory forms of hVDRR are sometimes treatable with supraphysiological doses of 1,25$(OH)_2D_3$; however, very high doses of 1,25-$(OH)_2D_3$ cannot be tolerated clinically, presumably because they will disrupt the normal homeostatic balance of non-nuclear receptor mediated 1,25$(OH)_2D_3$ responsive pathways. We envisioned that the recently reported VDR-1,25$(OH)_2D_3$ cocrystal structure could provide a basis to rationally design 1,25$(OH)_2D_3$ analogues capable of selectively complementing rickets-associated mutations such as VDR(R274L).[14]

Very recently, Gardezi et al. have shown that JK-1626−2, a known 1,25$(OH)_2D_3$ analogue, partially restores activity to VDR(R274L) with 5% of the potency of 1,25$(OH)_2D_3$ with VDR(wt).[4] This analogue was previously identified as a potent stimulator of PKC, a membrane receptor associated response of 1,25$(OH)_2D_3$.[15]

In this study we explore the use of structure-based design to rationally design 1,25$(OH)_2D_3$ analogues which are potent and selective activators of the rickets-associated mutant VDR(R274L). To provide insight into the binding mode of 1,25$(OH)_2D_3$ in the rickets-associated mutant, a computational site model representing the VDR(R274L) binding pocket was constructed in FLO/QXP,[16] based on the published X-ray coordinates of Moras et al. (Figure 1).[14] The

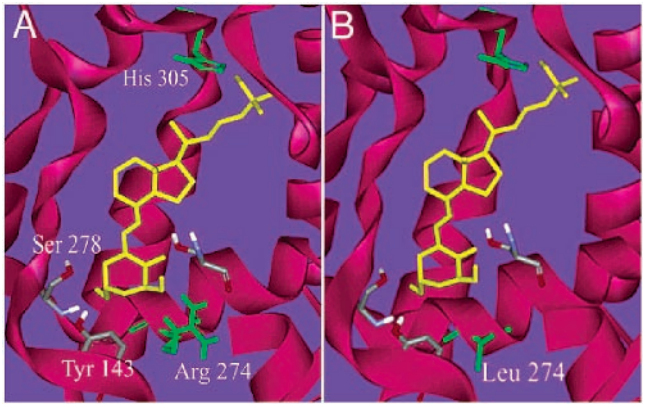

Figure 1. Comparison of the modeled structure of 1,25$(OH)_2D_3$ in VDR(R274L) with the wild-type ligand−receptor complex. (A) Structure of VDR(wt) with 1,25$(OH)_2D_3$ based on cocrystal structure. (B) Modeled structure of VDR(R274L) with 1,25$(OH)_2D_3$.

Arg274→Leu mutation results in the loss of a critical hydrogen bond that normally exists between the 1α-OH and the guanidine of Arg274 and opens a substantial hydrophobic "hole" within the ligand−receptor interface, adjacent to the position of the 1α-hydroxyl (Figure 1B).

The VDR(R274L) model suggests that hydrophobic functionality in the vicinity of the 1α-OH of 1,25$(OH)_2D_3$ may serve to replace the lost ligand−receptor hydrogen bond with a favorable "hydrophobic bond". Twenty candidate compounds were identified as having hydrophobic functionality of approximately the same size as the hole created by the Arg→Leu substitution and which could be easily derived

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Table 1.

	1,25D$_3$/ WT	1,25D$_3$/ R274L	SS-I/ R274L	SS-II/ R274L	SS-III/ R274L	SS-IV/ R274L
EC$_{50}$ (nM)	2.0 (±2.2)	>2000	N/A	116 (±6.7)	6.8 (±0.2)	24.2 (±1.2)
E_{ass} (kcal/mol)	−8.8	−7.3	−7.62	−8.82	−9.51	−10.1
max activity (RLU)	100	N/A	23	85	75	64

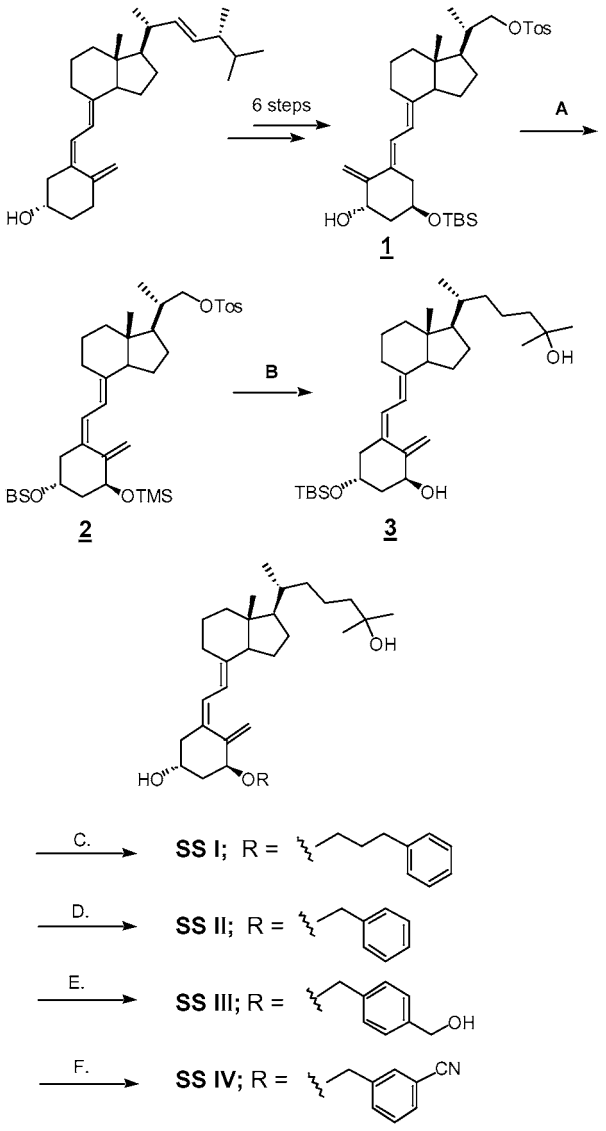
from commercially available reagents. The candidate compounds were subjected to comprehensive Monte Carlo docking simulations and the best three analogues, SS-II, SS-III, and SS-IV, were selected on the basis of their calculated apparent association energies for the VDR(R274L) site model (Eass). It is important to note that the calculations are not expected to represent the complex series of molecular events that represent ligand-dependent transcription; however, prior studies have suggested that similar hormone receptor models can be used as a reasonable guide to select compounds having a high propensity to be potent agonists.[17] The compound SS-I had a significantly lower (less negative) association energy presumably because the proposed A-ring substitution was too large for the binding pocket. This compound was also synthesized and evaluated as a negative control.

Our synthesis of the designed analogues was based on compound **1**, which was obtained from ergocalciferol following the six-step synthesis described by Hesse et al.[18] TMS protection of the 1α-hydroxyl allowed for its selective deprotection after installation of the biologically active C22-C25 TES-protected side chain. This allowed for the selective alkylation of 1α-hydroxyl with reagents selected by molecular design (Scheme 1).

The four analogues, SS-I to SS-IV, were evaluated for their ability to elicit a transactivation response in HEK293 cells transiently transfected with the mutant expression plasmid pSG5VDR(R274L) luciferase reporter (VDRE-luc), and control (pRLbasic, Promega) (Figure 2). The three designed analogues, SS-II, SS-III, and SS-IV, all show substantial activity in the Arg274→Leu mutant below 1 μM and are 17 to 286 times more potent than 1,25(OH)$_2$D$_3$ (EC$_{50}$ = >2000 nM). The analogues were able to restore 60 to 80% of the maximum inducible activity and 8 to 30% of the potency of 1,25(OH)$_2$D$_3$ with VDR(wt). The compound SS-I, designed as a negative control, has no detectable activity in the mutant below 2000 nM.

While the analogues SS-II, SS-III, and SS-IV are potent agonists for the mutant VDR(R274L), their utility as rickets-complementing analogues might be limited if they also strongly activated the rapid nonnuclear receptor mediated responses of 1,25(OH)$_2$D$_3$ and perturbed normal calcium homeostasis. The absence of a structure for the membrane-associated receptor precludes a structure-based approach to design ligands to be exclusive of membrane receptor associated actions. Because small substitutions at the 1-α position were previously shown to effect nuclear versus membrane selectivity,[15] we reasoned that the rather gross 1-α substitutions represented by SS-II, SS-III, and SS-IV might impart

Scheme 1. Synthesis of D$_3$ Analogs[a]



[a] Key: (a) TMS-imidazole, THF, rt 2 h; *hν*, PhMe, Et$_3$N, rt. (b) (TES(O)C(CH$_3$)$_2$(CH$_2$)$_2$Mg)$_2$Cu, THF/ET$_2$O, rt; KOH, EtOH, O °C. (c) NaH, DMF, PH(CH$_2$)$_3$Br; TBAF, THF, 40 °C. (d) NaH, DMF, BnBr; TBAF, THF, 40 °C. (e) NaH, DMF, (TMSO)CH$_2$C$_6$H$_4$-*o*-(CH$_2$)Cl; TBAF, THF, 40 °C. (f) NaH, DMF, *m*-CN-BnBr; TBAF, THF, 40 °C.

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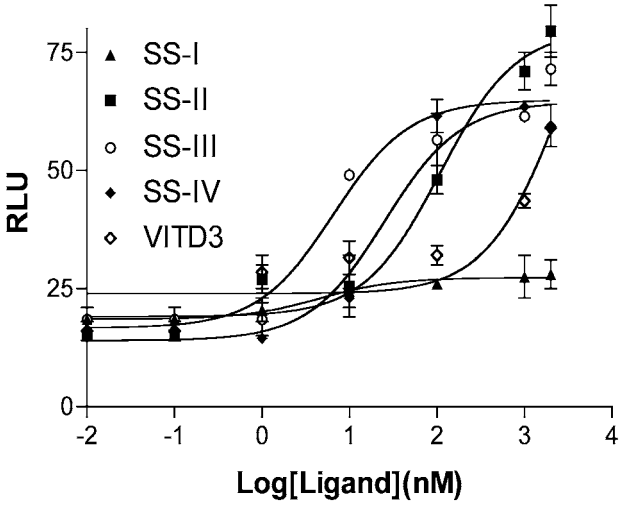

Figure 2. Transcription response of HEK293 cells transiently transfected with VDR-responsive luciferase promoter. Transactivation activity was determined by dual luciferase assay (Promega) and is reported as relative light units (RLU) normalized to the maximum inducible activity of $1,25(OH)_2$ D_3 in VDR(wt) having 100 RLU.

substantial selectivity against membrane-associated actions of $1,25(OH)_2D_3$ membrane-associated receptor. The designed analogues were assayed for their ability to stimulate Ca^{2+} influx via Voltage-Sensitive Calcium Channels (VSCC's) through the membrane-associated receptor using a radio-calcium influx assay under depolarizing conditions (high external K^+) (Figure 3).[19] All of the designed mutant-specific $1,25(OH)_2D_3$ analogues show significantly reduced activity toward ligand-mediated calcium influx compared to $1,25-(OH)_2D_3$ itself, suggesting that the designed mutant-selective analogues are capable of restoring function to the mutant nuclear receptor VDR(R274L) without substantially affecting the membrane-associated actions of $1,25(OH)_2D_3$.

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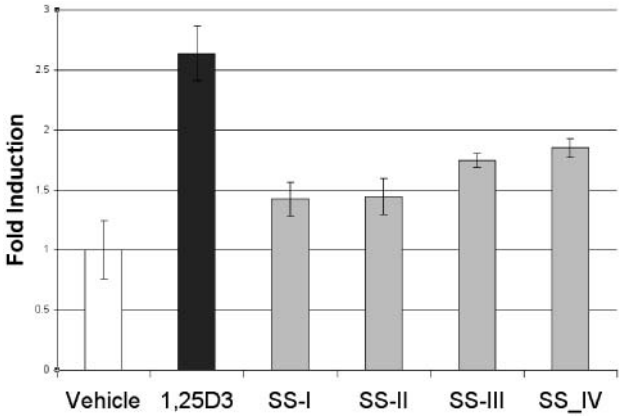

Figure 3. Ligand-dependent stimulation of $^{45}Ca^{2+}$ influx of MC3T3-E1 pre-osteoblastic cells under 132 mM K^+ with 20 nM ligand.

This work adds to a growing number of examples of small molecules which can restore function to mutationally impaired proteins associated with genetic disease. Clearly only a subset of mutations associated with genetic disease are amenable to small molecule molecular complementation. The rapid identification of mutant-compensating compounds of recoverable mutants therefore presents a new and unique challenge to the chemical community. The high-resolution structures of disease-associated proteins enable us to use structure-based design to rapidly identify mutant-compensating leads from existing ligands which may ultimately provide a new approach to the treatment of genetic disease.

Acknowledgment. pSG5hVDR was kindly provided by M. R. Haussler. We thank the National Institutes of Health (Grant No. RO1DK54257) for financial support.

Supporting Information Available: Synthesis and characterization of intermediates and analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0266931